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(54) Title: A PROCESS FOR INHIBITING SMOOTH MUSCLE CELL PROLIFERATION AND MOTILITY

(57) Abstract

A process of inhibiting smooth muscle cell proliferation and motility is provided. In accordance with the process, the activity of myosin light chain kinase is increased in the muscle cell by transfecting the cell with an expression vector containing a polynucleotide that encodes myosin light chain kinase or a catalytic portion thereof. A process of treating myointimal hyperplasia is also provided.

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A Process For Inhibiting Smooth Muscle Cell Proliferation And MotilityTechnical Field of the Invention

The field of this invention is cell proliferation and motility. More particularly, the present invention pertains to a process of inhibiting cell proliferation and motility by increasing the phosphorylation of myosin light chain in the cell.

Background of the Invention

The abnormal growth of smooth muscle cell lining the walls of blood vessels plays a key role in restricting blood flow. This abnormal growth of smooth muscle cell, generally known as intimal hyperplasia, contributes significantly to the incidence of restenosis associated with bypass surgery, balloon angioplasty, endarterectomy, atherectomy and laser recanalization. Intimal hyperplasia represents the normal healing response of the vessel wall that has gone awry. Division and migration of medial smooth muscle cell across the internal elastic lamina into the intima followed by further cell proliferation and the secretion of extracellular matrix are hallmarks of intimal hyperplasia. Growth factors and chemoattractants secreted by platelets attached to subendothelial collagen that is exposed due to the loss of the endothelium, growth factors found in the plasma and mechanical factors of the arterial circulation play critical roles in the genesis and development of intimal hyperplasia. Despite the clinical significance and the cost to society, no effective therapy has been developed to moderate intimal hyperplasia.

There is currently great interest in developing gene therapy approaches for treating intimal hyperplasia and other diseases. Gene therapy requires 3 elements: the availability of cloned genes, an expression vector containing the gene of interest and a mechanism for expressing the gene of interest in the target tissue. In many ways, the latter issue of obtaining targeted expression in a specific tissue or cell

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type, has been most problematic. Most of the gene therapy protocols that have been approved for human trials use retroviral or adenoviral vectors to deliver genetic material into cells. Viruses are the preferred vehicle for introducing exogenous genes into cells *in vivo* because virus infection can be very efficient compared to most transfection protocols. Still, it is difficult to target the infection of specific cells or a tissue *in vivo* using retroviral vectors.

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It would be best to perform the genetic manipulations *in vivo* using catheter based technology. This is the least invasive, most cost effective approach to gene therapy. However, all techniques have their drawbacks and this approach is no different. For instance, all transduction processes take time and even adenoviral expression may require occluding the circulation for an unacceptably long period of time (e.g., 10 minutes). Moreover, it has been suggested that the cells of the neointima are monoclonal in origin. This means that anything less than 100% transduction efficiency may be unsuccessful in preventing intimal hyperplasia. One solution to this problem is to use a selectable marker, such as the neomycin resistance gene, to delete the non-transduced smooth muscle cell using the glycoside G418. This, however, can only be done *in vitro*.

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Brief Summary of the Invention

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In one aspect, the present invention provides a process of inhibiting cell proliferation in a cell that contains myosin light chain. The process includes the step of increasing the phosphorylation of the myosin light chain in the cell. The phosphorylation of myosin light chain is increased by increasing the level of myosin light chain kinase activity in the cell.

The level of myosin light chain kinase activity is increased by transfecting the cell with an expression vector that contains a

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polynucleotide that encodes myosin light chain kinase. Preferred expression vectors are viral vectors such as a retroviral vector or a replication defective adenoviral vector. A process of the present invention has particular utility for inhibiting proliferation of vascular smooth muscle cells.

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In another aspect, the present invention provides a process of inhibiting cell motility in a cell that contains myosin light chain. Cell motility is inhibited using the same steps as set forth above in relation to the process of inhibiting cell proliferation. That is, the phosphorylation of myosin light chain is increased by increasing the levels of myosin light chain kinase activity in the cell, preferably by transfecting the cell with an expression vector that contains a polynucleotide that encodes myosin light chain kinase.

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In yet another aspect, the present invention provides a process of treating intimal hyperplasia of a blood vessel in an animal. In accordance with such a process, the level of myosin light chain kinase activity is increased in a smooth muscle cell of the blood vessel. The blood vessel can be an artery or a vein. The level of myosin light chain kinase is increased by transfecting the blood vessel with an expression vector that contains a polynucleotide that encodes myosin light chain kinase. Preferably, the blood vessel is transfected *ex vivo* and transplanted into the animal. The expression vector is preferably a retroviral vector or replication defective adenoviral vector.

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Brief Description of the Drawings

FIG. 1: Shows the domain Structure of MLCK.

FIG. 2: Shows a schematic presentation of the plasmid encoding tMK (pLNC-tMK).

FIG. 3: Shows the parent, control plasmid (pLNCX), which contains the neomycin resistance gene.

Detailed Description of the Invention

I. The Invention

5 The present invention discloses that cell proliferation and cell motility can be significantly inhibited by increasing the extent of phosphorylation of myosin light chain in the cell. The phosphorylation of myosin light chain is increased by increasing the activity level of an enzyme, or a catalytic portion thereof, that catalyzes the phosphorylation 10 of myosin light chain. Such an enzyme is myosin light chain kinase (MLCK).

The ability of MLCK to increase myosin light chain phosphorylation and, thus, cell proliferation and motility, can be used to treat intimal blood vessel hyperplasia using gene therapy techniques.

15 II. Inhibition of Cell Proliferation and Motility

In one aspect, therefore, the present invention provides a process of inhibiting cellular proliferation and/or cellular motility. In accordance with such a process, the phosphorylation of myosin light chain (MLC) is increased in the cell.

20 A myosin light chain is a subunit of the myosin II molecule. Myosin II is a member of the myosin super family of proteins (there are at least two members of this family). Myosin II has been found in all mammalian cells. Myosin II can have light chains that range in molecular weight from 17,000 to 27,000 daltons. All myosin II molecules 25 contain one set of two light chains of 18,000 to 20,000 daltons that get phosphorylated.

In accordance with a process of the present invention the phosphorylation of MLC is increased by increasing the activity of an enzyme that catalyzes MLC phosphorylation. A preferred such enzyme is myosin light chain kinase (MLCK). MLCK, as is well known in the art, is an enzyme that catalyzes the transfer of phosphate from ATP to MLC. 5 MLCK comprises numerous functional domains including a calmodulin binding domain, an ATP binding domain, a catalytic domain and a regulatory domain (see FIG. 1).

The level of MLCK activity in a cell is increased using standard 10 gene transfection techniques. Briefly, an expression vector that contains a polynucleotide that encodes MLCK is constructed and that vector is introduced into a target cell. MLC phosphorylation can also be increased by transfecting cells with an expression vector that contains a polynucleotide that encodes a portion of MLCK, which portion has the 15 ability to catalyze MLC phosphorylation. Such a catalytic portion is schematically shown in FIG. 1 and is referred to herein as truncated MLCK or tMK. The tMK gene was constructed by inserting a stop codon following lysine 793 of the MLCK gene. This prevents translation of the regulatory domain of the MLCK gene and results in a 20 constitutively active enzyme. The 1955 bp tMK gene was cloned into XbaI site of pBluescript II SK. The plasmid was then cut with NotI and the ends were filled in using Klenow fragment of *E. coli* DNA polymerase. The product was then cut with HindIII yielding a 2005 bp 25 piece of DNA containing the entire coding region of the tMK gene. The HindIII-blunt end fragment was cloned between the HindIII and HpaI sites of the pLNCX vector to form pLNC-tMK (see FIG. 2).

Eukaryotic expression vectors are well known in the art. A preferred expression vector for use in the present invention is a virus vector such as a retroviral or adenoviral vector. The use of virus vectors

for cell transfection is well known in the art. Virus vector-mediated cell transfection has been reported for various cells.

The major advantage of retroviral vectors is that they result in integration in the host genome, thereby resulting in long term expression of proteins in cells. Although the precise duration of expression is not known with certainty, there are reports of protein expression in vascular smooth muscle cells up to 6 months following retroviral transduction. On the negative side, retroviral infection is associated with low transduction efficiency of vascular smooth muscle cell when performed *in vivo*, partly because retroviruses most efficiently transduce rapidly replicating cells.

Adenoviruses can accommodate large genes (up to 8 kb). Also, it is possible to obtain high viral titres, thereby increasing their transduction efficiency, and efficiently infecting replicating and non-replicating cells. The disadvantages are that adenoviruses require a higher level of containment than retroviruses, they can result in cytopathology when used at high titre and adenovirus mediated gene transfer does not result in stable integration in the host genome.

Where the vector is an adenovirus vector, the viral vector of the present invention is preferably replication-defective. An adenovirus is rendered replication defective by deletion of the viral early gene region 1 (E1). An adenovirus lacking an E1 region is competent to replicate only in cells, such as human 293 cells, which express adenovirus early gene region 1 genes from their cellular genome. Thus, such an adenovirus cannot kill cells that do not express that early gene product. In a preferred embodiment, an adenovirus vector used in the present invention is lacking both the E1 and the E3 early gene regions. Techniques for preparing replication defective adenoviruses are well known in the art.

It is believed that any adenovirus vector can be used in the practice of the present invention. Thus, for example, an adenovirus vector can be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material for production of a replication-defective adenovirus vector.

A virus is engineered to contain a coding DNA sequence for use as a vector. Individual DNA sequences such as cDNAs that encode a gene product (e.g., MLCK) are inserted into the virus to create a vector construct.

In a preferred embodiment, a coding sequence for a gene product is introduced or incorporated into an adenovirus at the position from which the E1 coding sequences have been removed. However, the position of insertion within the adenovirus sequences is not critical to the present invention. A coding sequence can also be inserted in lieu of the deleted E3 region in E3 replacement vectors. Preferably, the E1 region of adenovirus is replaced by the coding DNA sequence or gene.

A replication deficient recombinant adenovirus that expresses tMK is constructed by homologous recombination in 293 cells, which supply *in trans* the necessary sequences to allow replication. Briefly, the tMK cDNA is excised from the pLNC-tMK plasmid by digestion with the restriction enzyme EcoRI and subsequently subcloned into the "shuttle plasmid" pACCMV.pLA. Proper orientation (5'->3') is confirmed by restriction fragment analysis after NcoI digestion. This subsequent plasmid (pShuttMK) includes, in order from 5' to 3', 0-1.3 map units of the sequence taken from the left end of the adenovirus 5 (Ad5) genome and, in place of Ela and part of Elb sequences (required for replication), the CMV immediate early promoter, the tMK sequences subcloned into the EcoR1 site of the pUC19 polylinker, the SV40 small T antigen intron and polyadenylation signal sequences and, finally, map

units 9 through 17 of Ad5. Equimolar amounts of pShuttleMK (0.2 µg) will be co-transfected, by cationic liposomes (Lipofectamine, GIBCO/BRL), into the 293 cell packaging line together with the plasmid pJM17 (0.8 µg). pJM17 sequences include the entire Ad5 genome (with a mutant E3 region), as well as ampicillin and tetracycline resistance sequences and a bacterial origin of replication. It is so large that it exceeds the DNA packaging limit for Ad5. Only adenoviral genomes formed by recombination between pJM17 and pShuttleMK are small enough to be packaged, thus selecting for E1⁻, E3⁻, replication deficient recombinant virions that include the tk expression sequences. 5 Recombinant plaques are validated by polymerase chain reaction, amplified in 293 cells, purified by double cesium chloride centrifugation and dialysis, and the titer determined.

10 AdCMVβGal is used in control experiments to characterize and optimize adenoviral gene expression in blood vessels. AdCMVβgal is a replication deficient, recombinant adenovirus that contains the 15 cytomegalovirus (CMV) early immediate promoter followed by the simian virus 40 nuclear localization signal and the *E. Coli lac Z* gene that encodes β-galactosidase (β-gal). This is a convenient vector for analyzing 20 gene expression since β-gal gene expression can be evaluated by 25 incubating tissue sections with x-gal reagent.

The resulting adenovirus vector is co-transfected into 293 cells together with a plasmid carrying a complete adenovirus genome to propagate the adenovirus. An exemplary such plasmid is pJM17. Co-transfection is performed in accordance with standard procedures well known in the art. By way of example, 293 cells are cultured in Dulbecco's modified Eagle's medium containing fetal calf serum. Confluent cultures are split the day before calcium phosphate co-transfection of plasmids. After addition of the DNA to the cells, the 30 cells are shocked (e.g., a 15% glycerol shock) to boost transfection

efficiency and the cells are overlaid with agar in DMEM containing fetal calf serum, penicillin, streptomycin sulfate, and other antibiotics or antifungal agents as needed. Monolayers are incubated until viral plaques appear (about 5-15 days).

5 These plaques are picked, suspended in medium containing fetal calf serum, and used to infect a new monolayer of 293 cells. When greater than 90% of the cells showed infection, viral lysates are subjected to a freeze/thaw cycle and designated as primary stocks. The presence of recombinant virus is verified by preparation of viral DNA from infected
10 293 cells, restriction analysis, and Southern blotting. Secondary stocks are subsequently generated by infecting 293 cells with primary virus stock at a multiplicity of infection of 0.01 and incubation until lysis.

15 The particular cell line used to propagate the recombinant adenoviruses of the present invention is not critical to the present invention. Recombinant adenovirus vectors can be propagated on, e.g., human 293 cells, or in other cell lines that are permissive for conditional replication-defective adenovirus infection, e.g., those which express adenovirus E1 gene products "in trans" so as to complement the defect in a conditional replication-defective vector. Further, the cells can be
20 propagated either on plastic dishes or in suspension culture, to obtain virus stocks thereof.

25 A viral vector used in a process of the present invention can also be a retroviral vector. An exemplary and preferred retroviral vector is pLNCX, a murine Maloney leukemia virus LTR-based vector (see FIG. 2). To transfect cells, specially prepared fibroblasts known as packaging cells must first be transfected with a retroviral construct.

Packaging cells are 3T3 cells that have been engineered so that they contain the viral gag, pol and env genes on separate plasmids.

Thus, these cells have all the components for making viral particles with the exception of the viral RNA. This is provided by the retroviral vectors because they contain sequences that result in their RNA being "packaged" into virus particles within the packaging cells. Packaging cells
5 have been super-transfected with the pLNCX and pLNC-tMK constructs. Briefly, packaging cells were plated at a density of 500,000 cells per 10 cm dish, transfected using the CaPO₄ method and selected in 0.8 mg/ml G418. Viral media from these cells have been used to infect 3T3 cells,
10 neuro 2A cells, epithelial cells, isolated smooth muscle cells and intact blood vessels.

In order to verify tMK expression, cells selected in G418 were labelled with ³²P and the myosin was immunoprecipitated from cell extracts using affinity purified antibodies to myosin. SDS PAGE followed by autoradiography showed a substantial increase in phosphorylation (0.96 mol PO₄/mol MLC₂₀) in NIH 3T3 cells infected
15 with tMK compared to control cells (0.20 mol PO₄/mol MLC₂₀) expressing neo.

A coding sequence for MLCK or a catalytic portion thereof can comprise introns and exons so long as the coding sequence comprises at least one open reading frame for transcription, translation and expression
20 of that polypeptide. Thus, a coding sequence can comprise a gene, a split gene or a cDNA molecule. In the event that the coding sequence comprises a split gene (contains one or more introns), a cell transformed or transfected with a DNA molecule containing that split gene must have means for removing those introns and splicing together the exons in the
25 RNA transcript from that DNA molecule if expression of that gene product is desired.

Where needed, a coding sequence of a virus vector construct is preferably operatively linked to an enhancer-promoter other than a virus

enhancer-promoter. A promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes. As used herein, the term "promoter" includes what is referred to in the art as an upstream promoter region, a promoter region or a promoter of a generalized eukaryotic RNA Polymerase II transcription unit.

Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer provides specificity of time, location and expression level for a particular encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. Unlike a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

As used herein, the phrase "enhancer-promoter" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art. As is also well known in the art, the precise orientation and location relative to a coding sequence whose transcription is controlled, is dependent *inter alia* upon the specific nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically

located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

5 An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a target cell. Exemplary and preferred enhancer-promoters are the cytomegalovirus (CMV) promoter, the Rous sarcoma virus (RSV) promoter, the EF1 α promoter, the muscle-specific creatine kinase (MCK) 10 enhancer or the 4F2 heavy chain enhancer (Zambetti et al., 1992; Yi et al., 1991 and Sternberg et al., 1988).

15 By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized. For example, selection of an enhancer-promoter that is active specifically in vascular smooth muscle permits tissue-specific expression of the gene product. Preferably a vascular smooth muscle specific enhancer-promoter is an endothelin promoter (see e.g., Lee et al., 1990 and Bloch et al., 1989) or a smooth muscle-actin promoter (see e.g., Foster et al., 1992 and Blank et al., 1992). Still further, selection of an enhancer-promoter that is regulated in response to a specific physiologic signal can 20 permit inducible gene product expression.

25 A coding sequence is operatively linked to a transcription terminating region. RNA polymerase transcribes an encoding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

Transcription-terminating regions are well known in the art. A preferred transcription-terminating region used in a virus vector construct of the present invention preferably comprises a polyadenylation signal of SV40, bovine growth hormone 3 or the protamine gene.

5 An expression vector can be used to transfect a cell either *in vitro* or *in vivo*. Where a cell is located *in vitro*, the cell is exposed to a culture medium that contains transfecting vectors. Typically, that medium is an aqueous medium that contains nutrients and other factors necessary for cell viability. Where the cell is located *in vivo*, the
10 expression vector is delivered into the organism such that the target cells are exposed to the expression vector.

15 A virus vector construct is typically delivered in the form of a pharmacological composition that comprises a physiologically acceptable carrier and the virus vector construct. An effective expression-inducing amount of a virus vector construct is delivered. As used herein, the term "effective expression-inducing amount" means that number of virus vector particles necessary to effectuate expression of a gene product encoded by a coding sequence contained in that vector. Means for determining an
20 effective expression-inducing amount of a virus vector construct are well known in the art. An effective expression-inducing amount of an adenovirus vector is typically from about 10^7 plaque forming units (pfu) to about 10^{15} pfu, preferably from about 10^8 pfu to about 10^{14} pfu and, more preferably, from about 10^9 to about 10^{12} pfu.

25 As is well known in the art, a specific dose level for any particular subject depends upon a variety of factors including the infectivity of the virus vector, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, and the severity of the particular disease undergoing therapy.

In a preferred embodiment, the cell being transfected is a vascular smooth muscle cell located in a blood vessel of an organism. A composition containing the vector is typically administered directly into the blood vessel in the vicinity of the smooth muscle cells to be transfected.

Two approaches have been used to target vascular smooth muscle cell using viruses. One approach is to infect cells in a section of vessel isolated by a double balloon catheter, *in situ*. Nabel and colleagues have described using retroviruses (Nabel et al., *Science*, 1990; and Nabel et al., *Science*, 1989) to express proteins in blood vessels. They have also used adenoviral vectors to express the herpes-virus thymidine kinase gene in pig iliofemoral arteries that were injured prior to infection. They have reported that subsequent systemic treatment with ganciclovir killed the smooth muscle cell expressing the thymidine kinase gene and prevented the proliferation of smooth muscle cell (Ohno et al., *Science*, 1994). Leiden and co-workers have also used a double balloon catheter to infect a section of injured vessel using an adenoviral vector with a constitutively active form of the retinoblastoma gene product (Chang et al., *Science* 1995). They reported that infection with this construct significantly reduced smooth muscle cell proliferation and neointimal formation in two models of vascular injury. The second approach has been to express proteins in endothelial or smooth muscle cell, *in vitro*, and then transplant the transfected cells into the vessel, *in vivo*. One publication using this approach reported that endothelial cells continued to express β -galactosidase reporter gene for 2-4 weeks following transplantation into pig iliac arteries denuded of endothelial cells (Wilson et al. *Science*, 1989). Clowes and colleagues (Lynch et al., *Proc. Natl. Acad. Sci. USA*, 1992) have infected vascular smooth muscle cells with a retroviral construct containing the gene for adenosine deaminase and demonstrated expression of enzyme activity for 6 months following transplantation into arteries denuded of endothelial cells.

III. Treatment of Intimal Hyperplasia

The arterial wall is a complex multicellular structure that plays important roles in inflammation, coagulation, and the regulation of blood flow. Vascular smooth muscle cells are located predominantly in the 5 arterial tunica media and are important regulators of vascular tone and blood pressure. These cells are normally maintained in a non-proliferative state *in vivo*. Arterial injury results in the migration of vascular smooth muscle cells into the intimal layer of the arterial wall where they proliferate and elaborate extracellular matrix components. 10 This neointimal smooth muscle cell proliferative response (intimal hyperplasia) has been implicated as important in the pathogenesis of atherosclerosis (Forrester et al., 1991; Ip et al., 1990).

Arterial injury following percutaneous balloon angioplasty of the coronary arteries leads to neointimal smooth muscle cell proliferation and clinically significant restenosis in 30-50% of patients (Schwartz et al., 15 1992; Liu et al., 1989). A large number of growth factors can induce the proliferation of vascular smooth muscle cells *in vitro* and *in vivo* (Schwartz et al., 1992; Liu et al., 1989; Fingerle et al., 1989; Libby et al., 1992). This redundancy in growth factor signaling pathways has led to 20 the suggestion that effective cytostatic therapies for vascular proliferative disorders should target nuclear cell cycle regulatory pathways rather than more proximal signal transduction molecules (Simons et al., 1992; Morishita et al., 1993; Barr and Leiden, 1994). Although a great deal 25 has been learned about the nuclear proteins that regulate cell cycle progression in cultured cells (Hollingsworth et al., 1993; Perry and Levine, 1993), relatively little is understood about the cell cycle regulatory pathways that control normal and pathologic smooth muscle cell proliferation *in vivo*.

Vascular smooth muscle cell (SMC) proliferation in response to 30 injury is an important etiologic factor in a number of vascular

proliferative disorders including atherosclerosis and restenosis following balloon angioplasty. Thus, a better understanding of the molecular mechanisms that regulate smooth muscle cell proliferation is important for the rational design of novel therapies for these diseases. The present invention describes the role of the MLC phosphorylation in regulating smooth muscle cell proliferation *in vivo*.

The present invention provides a process of treating intimal hyperplasia in an animal, including humans. In accordance with the process of the present invention, smooth muscle cells in a blood vessel undergoing intimal hyperplasia are manipulated to increase phosphorylation of myosin light chain in those muscle cells. The phosphorylation from myosin light chain in smooth muscle cells is increased by increasing the expression of myosin light chain kinase, or a catalytic portion thereof, in those cells. The level of myosin light chain kinase activity is increased by transfecting the smooth muscle cells with an expression vector that contains a polynucleotide that encodes myosin light chain kinase or a catalytic portion thereof, wherein the expression vector derives expression of the enzyme in the cell.

Transfection of vascular smooth muscle cells with an expression vector of the present invention can be accomplished *in vivo* or *ex vivo*. Where transfection occurs *in vivo*, an expression vector, as set forth above, is delivered to the vascular smooth muscle cells by inserting a catheter into a blood vessel undergoing intimal hyperplasia and delivering the vector in a pharmaceutical composition through the catheter. Means for delivering viral vectors to vascular smooth muscle cells using catheters is well known in the art and has been discussed above.

In a preferred embodiment, *ex vivo* gene transfer techniques are utilized. In accordance with such *ex vivo* techniques, vascular smooth muscle cells are transfected *in vitro* and the transfected cells transplanted

into a blood vessel of the organism. A more preferred embodiment, a vascular graft containing smooth muscle cells is transfected *in vitro*, and the vascular graft is then inserted into the organism at a desired location in a blood vessel undergoing intimal hyperplasia. In a specially preferred embodiment, the vascular graft is a venous graft. Venous grafts, 5 transfected *in vitro*, can be in the form of venous segments of intact tubes. Means for selecting and removing veins from animals for subsequent graft transplantation are well known in the art. Where vein segments are used for grafting, that segment is incubated with a media containing an expression vector as set forth above.

10 By way of example, incubation with retroviral media (DMEM supplemented with 10% fetal bovine serum and with antibiotics) lasts for 5 hours and is repeated on 3 consecutive days (culture days 2-4). Viral media is replaced with growth media following each 5 hour incubation period. Following the last incubation with viral media, the segments are 15 maintained in growth media for 2 days (culture days 5&6) to permit expression of the transduced genes. Infected segments are then selected by maintaining them for 8 days (culture days 7-14) in RPMI 1640 containing 10% fetal bovine serum, 2 mg/ml G418, antibiotics (selection media).

20 Following transfection of vein segments, those segments can be grafted into a target blood vessel using standard techniques well known in the art.

25 In a preferred embodiment, to facilitate graft re-implantation into the organism, *in vitro* transfection utilizes intact veins (e.g., intact tubes). In one embodiment, two holes are poked in the sides of 60 mm diameter petri dishes with a heated coat hanger. The holes are made about half way up the sides of the walls and are placed on opposite sides from each other. The male end of a plastic 3 way stopcock is then inserted in each

hole and glued. After the glue sets, a 2 inch piece of tygon tubing is slipped over the ends of the stopcock, connecting the two stopcocks. Liquid Sylgard is poured into the petri dish so that the stopcocks are covered with 2-3 mm of Sylgard and placed under a slight vacuum
5 overnight to harden. The vacuum removes any bubbles in the sylgard and the tubing prevents the stopcocks from getting plugged. When the Sylgard sets, a scalpel blade is used to trim out a trough in the Sylgard that is about 1 cm wide. The trough is fashioned so that it extends beyond the ends of the tygon tubing, which is removed along with the
10 Sylgard. The distance between the tips of the stopcocks accommodates vessels that are slightly longer. Casting the stopcocks into the Sylgard prevents leaks from occurring at the sites that the stopcocks are glued to the plastic. Moreover, the entire apparatus, including the lid, can be gas sterilized. Most importantly, the trough minimizes the amount of viral
15 media needed to infect the vessels.

To culture blood vessels, intact veins with all side branches tied off are slipped over the ends of the stopcock and tied tightly to prevent any leaks between the vessel and the stopcock. Care is taken to gently stretch the vessel to the *in vivo* length. The trough is then filled with media. The lumen of the vessel is also filled with media, the stopcocks turned to the off position, the lid replaced on the petri dish and the entire apparatus placed in an incubator. In this way, the vessel is bathed in media from the luminal and adventitial sides and the vessel stays patent. The media in the trough and the vessel are replaced periodically
20 as needed. All manipulations are performed in a laminar flow hood under sterile conditions.
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Alternatively, veins are connected to a Cellco, Inc. Cellmax^R perfusion system. This perfusion system was originally designed to perfuse packaging cells grown in hollow fiber culture systems. This
30 perfusion system appears to increase the viral titre compared to cells

grown in culture plates. It contains 4 peristaltic micropumps that are used to circulate media through artificial capillary cell culture systems. The media is oxygenated through gas permeable tubing and the entire apparatus can be gas sterilized. This perfusion system, which is designed to go inside a tissue culture hood, is ideal for maintaining the low flow rates needed to perfuse intact blood vessels, which are attached to the perfusion apparatus through the 3 way stopcocks.

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Vessels mounted in culture dishes are placed in 5% CO₂, 37°C overnight. The vessels are infected by replacing the growth media with 10 viral media from packaging cells transfected with an expression vector on both the outside and the inside of the vessels. Following selection, the vessels are cultured for a total of 14 days.

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Some veins (from 7-10 patients) are used to extract RNA to perform RT-PCR using actin, neo and tMK primers to establish that the infected vessels are expressing neo and tMK. Others (from 7-10 patients) are fixed, *in situ*, and processed for histological examination (i.e., gross appearance and quantitation of intimal area). These sections are compared to sections prepared from pieces of the same vessels that were fixed on day 1 and to sections from another group of veins (from 7-10 patients) that were cultured under identical conditions without infection or selection.

The penetration and infection of medial smooth muscle cell in blood vessels appears to be greatly affected by the endothelium. In the presence of the endothelium, endothelial cells are transduced and β -gal expression is rarely seen in the medial smooth muscle cell. In contrast, β -gal expression is seen throughout the media when vessels are transduced with β gal expressing adenoviruses in the absence of the endothelium. Therefore, vessels are opened longitudinally and denuded of endothelial cells by gently scraping them off with a Q-tip. Studies by

both the Leiden (Chang et al., *Science*, 1995) and Dzau (Morishita et al., *Proc. Natl. Acad. Sci. USA*, 1993) groups have demonstrated that the endothelial cells grow back rapidly and that intimal hyperplasia can be prevented for short periods of time by genetically manipulating the smooth muscle cell.

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The following Examples illustrate particular embodiments of the present invention and are not limiting of the claims and specification in any way.

EXAMPLE 1: General Techniques

10 a. Isolation and infection of vascular smooth muscle cell: Vascular smooth muscle cells are isolated, dispersed and cultured for 10-14 days. The cells are split and infected with retroviral media from packaging cells transfected with an expression vector (e.g., LNCX and LNC-tMK) (see FIGs. 2 and 3). The cells are incubated with viral media for 5 hrs a day for 3 consecutive days. They are then selected in G418³ (0.6 mg/ml) in M199 media supplemented with 10% FBS for 8 days. Non-transfected pulmonary smooth muscle cell are also treated with G418 at the same time to make sure that all of the uninfected cells die before 8 days.

15 b. Verification of tMK and neo expression in infected smooth muscle cell: The transcription of tMK RNA in the infected cells is examined by RT-PCR. PCR primers for actin, neo and tMK are used and RNA is extracted from 10⁶ uninfected, neo and tMK infected cells for PCR analysis. Actin primers function as the positive control for the PCR reactions. The neo primers should yield a product in both neo and tMK cells and the tMK primers should yield a product only in the tMK infected cells. PCR amplification in the absence of reverse transcriptase and in the absence of DNA serves as negative controls for each set of primers.

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Expression of tMK in active form is examined by immunoprecipitating myosin from 1.5×10^6 neo and tMK cells labelled with ^{32}P using an affinity purified antibody to tracheal smooth muscle myosin. The stoichiometry of MLC-P in these cells is determined by performing IEF PAGE on the immunoprecipitated myosin.

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c. Analysis of growth characteristics: The growth rate of uninfected and infected smooth muscle cell is estimated by measuring the doubling time and ^3H -thymidine incorporation into DNA. To quantify the doubling time, 4×10^5 cells are seeded in 6 well plates and allowed to attach in medium M199 supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin. The cells are then synchronized in G0 by incubation in serum free M199 for 48 hours, trypsinized and counted. The same number of cells (50,000) are then seeded in each well of 6-well plates and stimulated to proceed through the cell cycle by growing them in M199 media supplemented with 10% FBS. The cells are then trypsinized and counted every 24 h for 3 days.

Thymidine incorporation is used as a measure of DNA synthesis. Briefly, isolated smooth muscle cell are grown in 24 well dishes. Sub-confluent cells (30,000 per well) are serum starved for 48 hours in serum free M199. Following synchronization, serum free media is replaced with M199 containing 10% FBS and ^3H -thymidine ($1.5 \mu\text{Ci}$ per well). Some cells are treated with ^3H -thymidine in serum free M199 and these cells serve as a control. After 16 hrs, excess thymidine is removed by washing twice with ice cold FBS and fixed in 0.5 ml of ice cold 10% trichloroacetic acid. The acid is then removed and replaced with 0.8 ml of 0.2 N NaOH. The radioactivity in the NaOH is then quantitated by liquid scintillation counting.

EXAMPLE 2:

Porcine external jugular vein segments that were cultured for 14 days (culture control), segments that were infected with the LNCX vector and selected in G418 (vector control) and segments infected with LNC-tMK and selected in G418 were studied. Three types of procedures have been performed on these vessels segments: (a) histology prior to implantation to evaluate the effect of culturing and genetically manipulating the blood vessels on intimal hyperplasia, (b) PCR analysis prior to implantation to evaluate expression of the genes introduced by the viruses and (c) histology on vessel segments 3-6 weeks following re-implantation into the donor pig. Staining with H&E and Gamori's trichrome have demonstrated the presence of viable smooth muscle cell in vessels cultured for 14 days. Moreover, these culture conditions result in the formation of a significant neointima. These results are consistent with those previously reported by Soyombo et al. for human saphenous veins maintained in culture under similar conditions.

PCR technology was used to investigate gene expression following infection of the blood vessels. RNA was isolated from control, LNCX and LNC-tMK infected vessel segments and first strand cDNA synthesis reactions were performed with 10 µg of each RNA. Specific primers for amplification of smooth muscle actin were 5'-
CACTGGCATTGTCATGGACT-3' (SEQ ID NO:1) and 5'-
CTTCATCTTCATGGTGCTCC-3' (SEQ ID NO:2) (predicted size of
this PCR product is 541 bp). The primers for the phosphotransferase
that confers neomycin resistance were 5'-
CTGAATGAACTGCAGGACGAGG-3' (SEQ ID NO:3) and 5'-
CGCCAAGCTTCTTCAGCAATATC-3' (SEQ ID NO:4) (predicted size
= 532 base pairs). Primers for tMK were designed to specifically identify
tMK expression from the vector and to not detect endogenous MLCK.
Thus, one primer, 5'-ATCGAATTCTGCAGC-3' (SEQ ID NO:5),
hybridized to a sequence of pBluescript II SK retained in the tMK vector

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while the other primer, 5'-CTACACATTGCACGAGC-3' (SEQ ID NO:6), hybridizes within the tMK gene. The predicted size of this PCR product was 440 base pairs. A PCR product of the appropriate size using tMK primers is only seen in vessel segments infected with LNC-tMK.

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Examination of blood vessels cultured for 14 days show that tMK expression followed by G418 selection dramatically decreases the intimal mass. However, this may be related to G418 selection. To evaluate this possibility, the intimal thickness in 3 types of vessel segments was quantitated: culture control, vector control and tMK expressing vein segments. The data show that infection with either construct followed by G418 selection results in a pronounced reduction in intimal thickness. This is potentially very significant because it suggests that non-transduced cells were eliminated by G418 selection and that the remaining smooth muscle cell in the tMK infected vessels will undergo *controlled* growth, thereby limiting intimal proliferation, when they are re-implanted into pigs.

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Preliminary data have also been obtained from vessel segments that were removed 6 weeks after they were re-implanted in pigs. A control segment was a piece of left external jugular vein that was removed and immediately re-implanted into the carotid. This segment shows a very high degree of intimal hyperplasia. Histologically, it was very similar to a segment that was cultured for 14 days and a segment infected with LNCX and selected in G418 when they were removed 6 weeks after re-implantation. In contrast, the vein segment infected with tMK shows a larger caliber lumen and dramatically less intimal proliferation. Data from two other animals supports the hypothesis that tMK expression prevents or retards intimal proliferation in pigs.

In summary, they show that (a) veins can be maintained in culture for 14 days, (b) our culture conditions result in intimal hyperplasia, *in vitro*, as previously described (Soyombo et al., *Am. J. Path.*, 1990), (c) expression of tMK from retroviral vectors occurs in blood vessels using 5 PCR technology and (d) tMK expression inhibits intimal hyperplasia in vein segments that were removed 6 weeks following re-implantation.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: de Lanerolle, Primal Eton, Darwin
- (ii) TITLE OF INVENTION: A Process for Inhibiting Smooth Muscle Cell Proliferation and Motility

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACTGGCATT GTCATGGACT

20

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTCATCTTC ATGGTGCTCC

20

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGAATGAAC TGCAGGACGA GG

22

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCCAAGCTT CTTCAGCAAT ATC

23

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:

26

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATCGAATTCC TGCAGC

16

- (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "primer"

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTACACATTG CACGAGC

17

WHAT IS CLAIMED IS

1. A process of inhibiting cell proliferation in a cell that contains myosin light chain comprising increasing the phosphorylation of the myosin light chain in the cell.
2. The process of claim 1 wherein the phosphorylation of myosin light chain is increased by increasing the activity of myosin light chain kinase in the cell.
3. The process of claim 2 wherein the activity of myosin light chain kinase is increased by transfecting the cell with an expression vector that contains a polynucleotide that encodes myosin light chain kinase, or a catalytic portion thereof.
4. The process of claim 3 wherein the expression vector is a viral vector.
5. The process of claim 4 wherein the viral vector is a retroviral vector or replication defective adenoviral vector.
6. The process of claim 1 wherein the cell is a vascular smooth muscle cell.
7. A process of inhibiting cell motility in a cell that contains myosin light chain comprising increasing the phosphorylation of the myosin light chain in the cell.
8. The process of claim 7 wherein the phosphorylation of myosin light chain is increased by increasing the activity of myosin light chain kinase in the cell.

9. The process of claim 8 wherein the activity of myosin light chain kinase is increased by transfecting the cell with an expression vector that contains a polynucleotide that encodes myosin light chain kinase or a catalytic portion thereof.

10. The process of claim 9 wherein the expression vector is a viral vector.

11. The process of claim 10 wherein the viral vector is a retroviral vector or replication defective adenoviral vector.

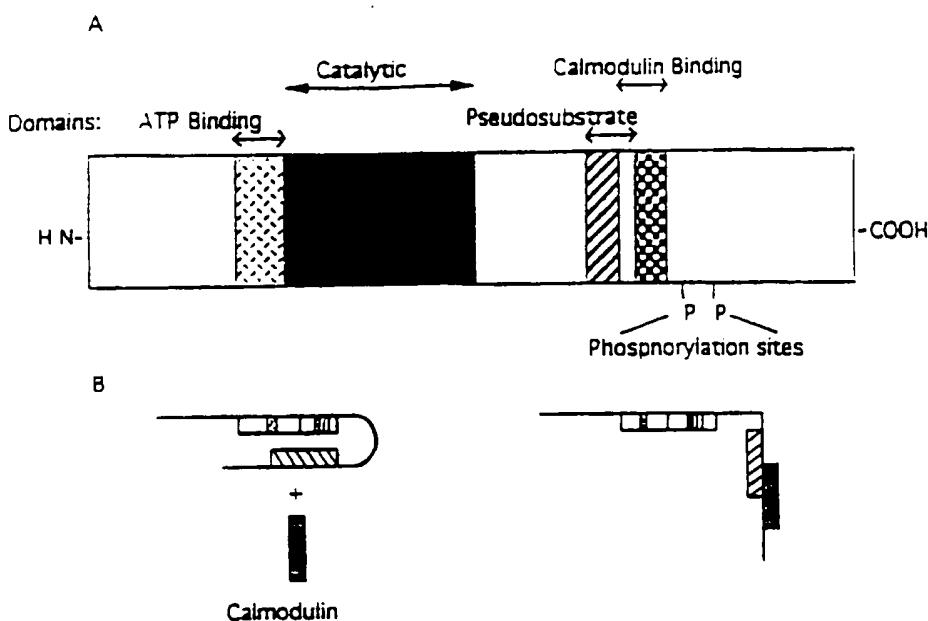
12. The process of claim 7 wherein the cell is a vascular smooth muscle cell.

13. A process of treating intimal hyperplasia of a blood vessel in an animal comprising increasing the activity of myosin light chain kinase in a smooth muscle cell of the blood vessel.

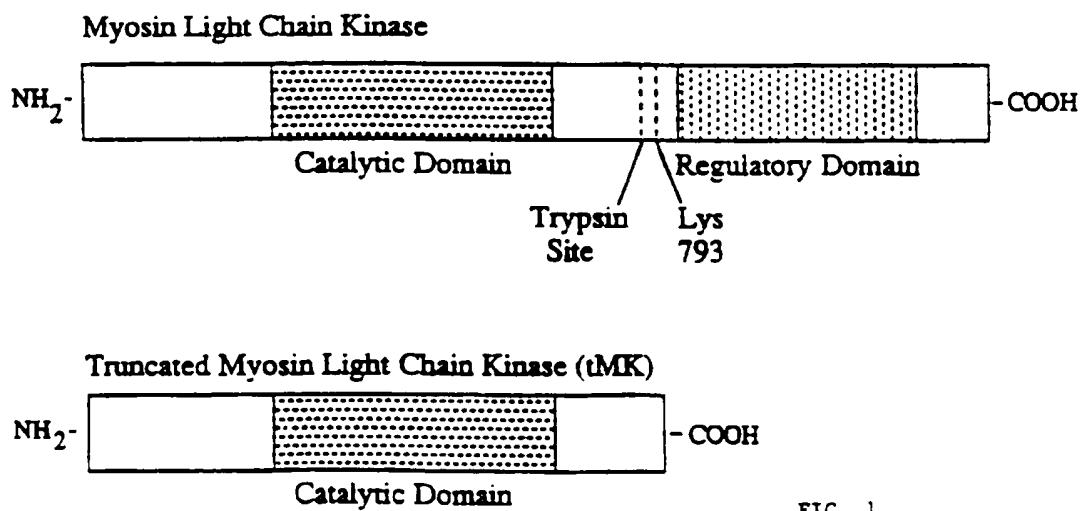
14. The process of claim 13 wherein the activity of myosin light chain kinase is increased by transfecting the cell with an expression vector that contains a polynucleotide that encodes myosin light chain kinase or a catalytic portion thereof.

15. The process of claim 14 wherein the expression vector is a replication-defective retroviral vector or adenoviral vector.

1/3
MYOSIN LIGHT CHAIN KINASE



**Domain Structure of
Myosin Light Chain Kinase**

FIG. 1

2/3

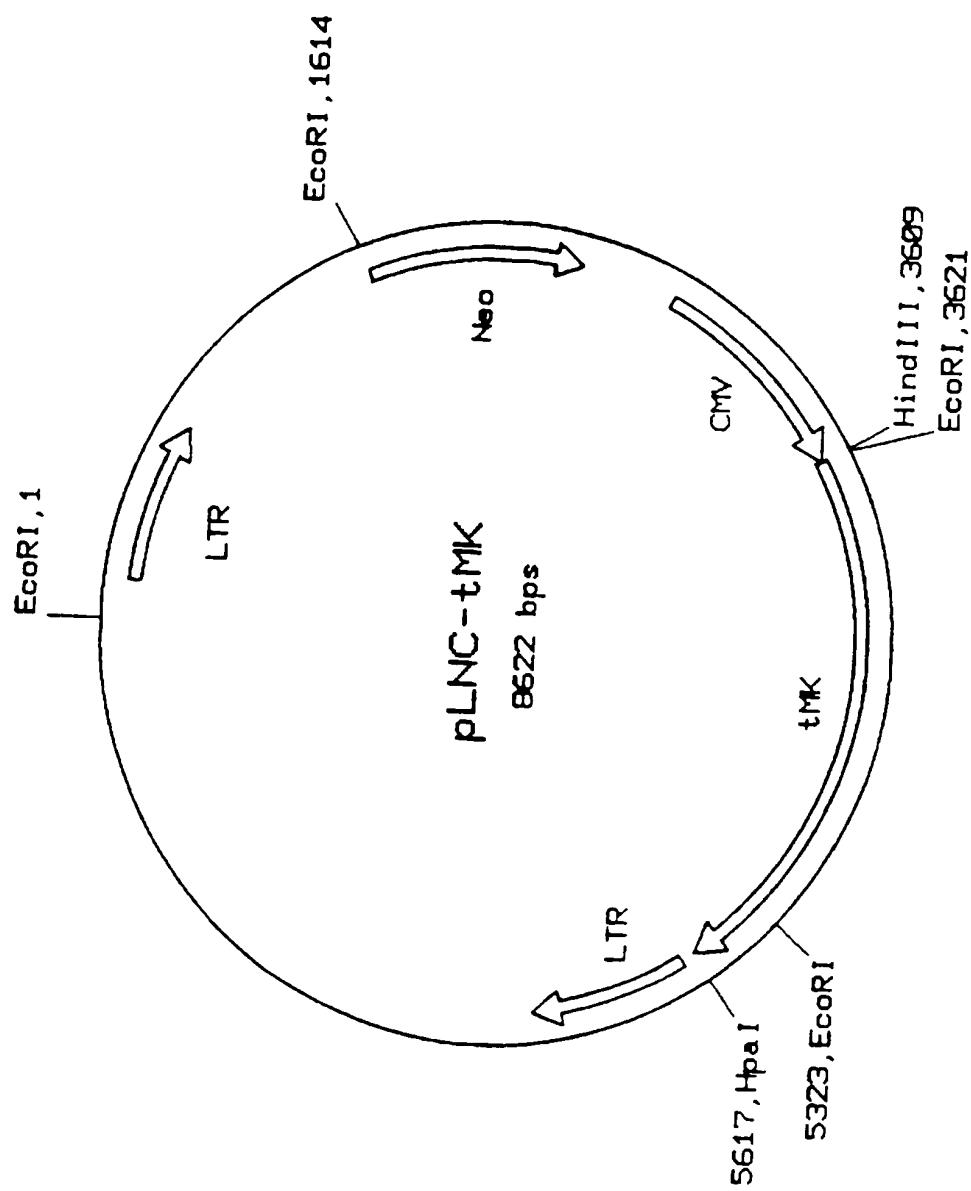


FIG. 2

3/3

